Real-time quantification of amino acids in the exhalome by secondary electrospray ionization-mass spectrometry: a proof-of-principle study

García-Gómez, Diego ; Gaisl, Thomas ; Bregy, Lukas ; Cremonesi, Alessio ; Sinues, Pablo Martinez-Lozano ; Kohler, Malcolm ; Zenobi, Renato

Abstract: BACKGROUND: Amino acids are frequently determined in clinical chemistry. However, current analysis methods are time-consuming, invasive, and suffer from artifacts during sampling, sample handling, and sample preparation. We hypothesized in this proof-of-principle study that plasma concentrations of amino acids can be estimated by measuring their concentrations in exhaled breath. A novel breath analysis technique described here allows such measurements to be carried out in real-time and noninvasively, which should facilitate efficient diagnostics and give insights into human physiology. METHODS: The amino acid profiles in 37 individuals were determined by ion-exchange HPLC in blood plasma and simultaneously in breath by secondary electrospray ionization coupled to high-resolution mass spectrometry. Participants were split into training and test sets to validate the analytical accuracy. Longitudinal profiles in 3 individuals were additionally obtained over a 12-h period. RESULTS: Concentrations of 8 slightly volatile amino acids (A, V, I, G, P, K, F, Orn) could be determined in exhaled breath with a CV of <10%. Exhalome validation studies yielded high accuracies for each of these amino acids, on average only 3% less compared to plasma concentrations (95% CI ±13%). Higher variations were found only for amino acids with a low plasma concentration. CONCLUSIONS: This study demonstrates for the first time that amino acids can be quantified in the human breath and that their concentrations correlate with plasma concentrations. Although this noninvasive technique needs further investigation, exhalome analysis may provide significant benefits over traditional, offline analytical methods.

DOI: https://doi.org/10.1373/clinchem.2016.256909

Originally published at:
García-Gómez, Diego; Gaisl, Thomas; Bregy, Lukas; Cremonesi, Alessio; Sinues, Pablo Martinez-Lozano; Kohler, Malcolm; Zenobi, Renato (2016). Real-time quantification of amino acids in the exhalome by secondary electrospray ionization-mass spectrometry: a proof-of-principle study. Clinical Chemistry, 62(9):1230-1237.
DOI: https://doi.org/10.1373/clinchem.2016.256909

Research Article | Article

Real-Time Quantification of Amino Acids in the Exhalome by Secondary Electrospray Ionization–Mass Spectrometry: A Proof-of-Principle Study

Diego García-Gómez, Thomas Gaisl, Lukas Bregy, Alessio Cremonesi, Pablo Martinez-Lozano Sinues, Malcolm Kohler, Renato Zenobi

DOI: 10.1373/clinchem.2016.256909 Published September 2016

Abstract

BACKGROUND: Amino acids are frequently determined in clinical chemistry. However, current analysis methods are time-consuming, invasive, and suffer from artifacts during sampling, sample handling, and sample preparation. We hypothesized in this proof-of-principle study that plasma concentrations of amino acids can be estimated by measuring their concentrations in exhaled breath. A novel breath analysis technique described here allows such measurements to be carried out in real-time and noninvasively, which should facilitate efficient diagnostics and give insights into human physiology.

METHODS: The amino acid profiles in 37 individuals were determined by ion-exchange HPLC in blood plasma and simultaneously in breath by secondary electrospray ionization coupled to high-resolution mass spectrometry. Participants were split into training and test sets to validate the analytical accuracy. Longitudinal profiles in 3 individuals were additionally obtained over a 12-h period.

RESULTS: Concentrations of 8 slightly volatile amino acids (A, V, I, G, P, K, F, Orn) could be determined in exhaled breath with a CV of <10%. Exhalome validation studies yielded high accuracies for each of these amino acids, on average only 3% less compared to plasma concentrations (95% CI

±13%). Higher variations were found only for amino acids with a low plasma concentration.

CONCLUSIONS: This study demonstrates for the first time that amino acids can be quantified in the human breath and that their concentrations correlate with plasma concentrations. Although this noninvasive technique needs further investigation, exhalome analysis may provide significant benefits over traditional, offline analytical methods.

Determination of the concentrations of amino acids is often performed in medicine, including in neonatal screening programs for detecting metabolic disorders, or in adults to monitor disease activity and treatment and for differential diagnosis. In addition to their everyday clinical use, amino acids are also emerging as biomarkers for early detection of a number of common and complex diseases, because of their central role in human metabolism. Accordingly, plasma free amino acid (PFAA)[6] profiles have become a powerful tool for diagnosis over the past few years. The predictive value of amino acid profiles have been demonstrated for diseases such as cancer (**1**, **2**), type-2 diabetes (**3–5**), and Alzheimer disease (**6**). Among PFAAs, branched-chain amino acids (BCAA), i.e., L, I, and V, have been studied more extensively because of their unique skeletal muscle metabolism. Strong evidence has been found linking BCAA to type-2 diabetes (**7–9**) and pancreatic adenocarcinoma (**10**).

Ion-exchange HPLC (IE-HPLC) followed by ninhydrin derivatization for UV detection is the current gold standard for analyzing amino acids in body fluids. However, this method is invasive, time-consuming, and lacks specificity, because metabolites and drugs other than amino acids can react with ninhydrin and coelute during the measurement process. IE-HPLC analysis is also complex, requiring a skilled operator, and generates a substantial amount of solvent waste. Most importantly, to be successful, this method requires robust and standardized sampling, sample handling, and sample preparation.

Analysis of exhaled breath, on the other hand, provides instant results, and may thus offer a simple alternative to traditional laboratory based methods. In this sense, breath analysis is gaining attention as a novel technique for clinical purposes such as diagnostics (**11**) and pharmacokinetics (**12**). Breath analysis can detect differences between individual “breathprints” which are reasonably stable over time (**13**), can be used to follow diurnal metabolic patterns (**14**, **15**), and is capable of quantifying hitherto unknown compounds in human breath (**16**, **17**). It also has been proposed for the detection of biomarkers to diagnose lung and sleep diseases (**18**, **19**). In all of these applications, breath analysis is usually thought of as being restricted to volatile or semivolatile compounds that cross from the blood to the breath via the alveolar compartment (**20**). However, a new generation of instruments that employ very sensitive high-resolution mass spectrometry (HRMS) methods, including proton-transfer-reaction TOF (**21**) MS or secondary electrospray ionization (SESI)–Orbitrap MS, (**22–24**) now reach limits of detection in the low parts-per-trillion by volume (pptv) range (**25**), which enables the detection of only partially volatile compounds. This applies to several PFAAs whose reference interval plasma concentrations are in the range of 10–1000 μmol/L (**25–27**) and whose Henry's law constants for aqueous solutions are as high as 10^5–10^6 mol · m^{-3} · Pa^{-1} (**28**), which results in expected breath concentrations of only around 1–10 pptv.

The aim of this work was to investigate whether amino acids, including BCAA, could be detected in exhaled breath by means of SESI-HRMS. Furthermore, we hypothesized that if even minute concentrations of amino acids can be quantified in breath via SESI-HRMS, this may allow a correlation with plasma concentrations to be established. We suggest that determination of amino acids in breath could become an interesting alternative diagnostic tool compared to traditional plasma amino acid studies.

Materials and Methods

STUDY DESIGN AND PARTICIPANTS

The amino acid profile was synchronously determined by IE-HPLC (blood plasma) and SESI-HRMS (exhalome) in 37 individuals without any known metabolic disease. A power calculation was performed to estimate the required sample size. This calculation used an α of 0.05 and a β of 0.2, and suggested that 12 individuals would be required to detect a mean alanine concentration difference of 50 μmol/L between the IE-HPLC analyzed plasma amino acid concentration and the SESI-HRMS exhalome analysis. A 50 μmol/L alanine threshold is smaller than interpersonal differences for this amino acid and was chosen based on our previous experience with this technology (**29**). Subsequently, participants were split into training (n = 25) and test (n = 12) sets, with no significant differences identified in their baseline characteristics (sex, age, body-mass-index, see **Table 1** in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org /content/vol62/issue9). The training set was derived from a cystic fibrosis cohort and used to develop the predictive model. The test set (healthy controls) was used to provide a prospective independent performance validation of the model.

REAL-TIME BREATH ANALYSIS

The methods used for sampling, processing, and analyzing the data in this study have been published in detail elsewhere (**19**). In short, study requirements for participants were to refrain from consumption of alcohol and caffeine or use of tobacco and chewing gum 1 hour before assessment. All candidates were in a fasting state before measurement (**19**). For SESI-HRMS, a standardized protocol was applied to all participants to exclude the influence of breathing maneuvers on exhaled compounds and to keep artifacts to a minimum level (**19**, **30**, **31**). Exhalations via a mouthpiece at a level of 10 mbar lasting 15 s were repeated 6 times. Exhaled breath was conducted via a heated (90 °C) Teflon tube connected to the curtain gas port of a TOF mass spectrometer (**32**) (TripleTOF 5600, AB Sciex) and analyzed in real time (see online Supplemental Fig. 1). The last 6 s of each exhalation (approximately 15 s) were considered for analysis, thus excluding the initial dead volume (air from the upper airways which is inhaled and does not take part in the gas exchange) (**19**). Signals obtained for each amino acid, by integrating the HRMS baseline resolved peaks, were normalized against the signal of an internal standard, D_4-alanine (integrated over the same 6 s). This isotopically labeled internal standard was added to the nanoelectrospray solution and proved to be useful for correction of the

instrumental shift. For SESI-HRMS/MS analysis, product ion scan experiments were run with a collision energy of 30 (15) V and an isolation window of 1 Da.

PLASMA ANALYSIS

Venous blood samples of 10 mLwere obtained within 5 min of breath analyses via vacutainer blood collection tubes (BD) coated with lithium-heparin (17 IU/mL). Blood plasma samples were frozen to −80 °C and stored for further analysis. The amino acids analysis was performed by means of IE-HPLC in an accredited diagnostic laboratory using routine procedures and 2 identical IE-HPLC setups (HPLC 1 and 2). Briefly, 200 μL plasma samples were mixed with 20 μL of a sulfosalicylic acid solution (40% w/v). The precipitated proteins were removed by centrifugation at 5900*g* and 10 °C for 10 min. Then the supernatant was diluted with an equal volume of citrate loading buffer, and 50 μL of the solution was injected on a Biochrom 30 Plus amino acid analyzer (Biochrom Ltd). Amino acids were separated by IE-HPLC using a lithium high-performance physiological column (Biochrom Ltd) followed by postcolumn derivatization of the eluting amino acids with ninhydrin and detection of the derivatized amino acids at 570 nm and 440 nm (**33**). In total 46 amino acids could be separated over 153 min by means of a step-gradient elution using 5 distinct lithium citrate buffers of different ionic strength (from 0.20 mol/L to 1.65 mol/L) and pH (from 2.80 to 3.55). The column temperature was also varied step-wise during the run from initial 33 °C to final 78 °C, whereas the flow was kept constant at 20 mL/h. The quantification was performed by means of external calibrators for each amino acid using the EZChrom Elite software (Agilent Technologies Inc). The amino acids were identified according to the retention time and the ratio of the area between the 2 wavelengths (570 nm and 440 nm).

STATISTICS

Breath signals from the training set (n = 25) were normalized against D_4-alanine and used to build a calibration curve for each amino acid (normalized breath signal vs plasma concentration) by means of Passing–Bablok regression (**34**).

These signals resulted from averaging the last 6 scans (accumulation time per scan = 1 s) of each exhalation (i.e., reflecting mostly the end-tidal fraction). In all cases the same number of scans (i.e., 6) was used for the mean of the breath mass spectra including the internal standard.

Afterward, normalized breath signals from the test set (n = 12) were used to calculate plasma concentrations, by means of the calibration curves previously obtained, and compared with the values obtained from plasma analysis using Bland–Altman plots as a way of assessing agreement between the 2 clinical methods.

QUANTIFICATION OF VAPOR CONCENTRATION

To check our hypothesis of amino acids being exhaled at the low pptv range, alanine was quantified in breath by a method recently developed by Aernecke et al. (**35**) showing “a rapid, near-real-time capability to quantitatively measure low-vapor-pressure compounds.” Briefly, the method is based on

the generation of known vapor concentrations, calculated from the Clausius–Clapeyron equation, from a solid at different temperatures. A scheme of the setup is shown in **Fig. 1**. The mass spectrometer was the same used for breath analysis. Tested temperatures were 50.0 °C, 75.0 °C, 100.0 °C, 125.0 °C, and 150.0 °C (±0.1 °C). Vapor concentrations generated by these temperatures were calculated from the Antoine equation using the following values tabulated for alanine: A= 11.81037, B = 5776.202, and C= −34.143. All experiments were carried out by triplicate and averaged. Final concentrations (**Fig. 1**) were the result of averaging multiple breath responses.

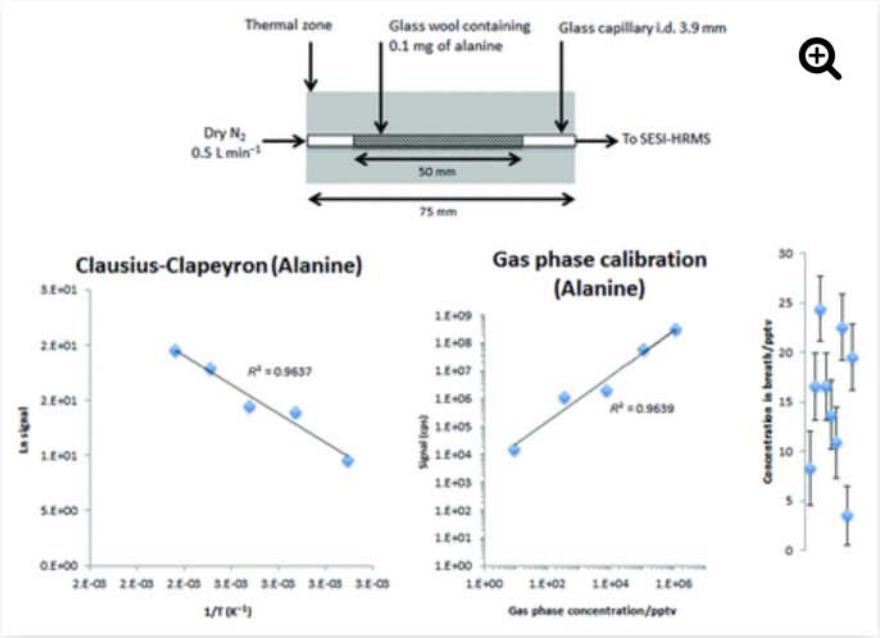

Fig. 1. **Download figure | Open in new tab | Download powerpoint**

Gas-phase quantification of alanine in breath according to the Aernecke et al. method *(34)*.

ETHICS

An independent ethics authority (Cantonal Ethics Committee Zurich, Switzerland) approved this study with the number KEK-ZH-Nr. 2014–0076. Written informed consent was obtained from each participant before participation in the study.

Results

DETECTION AND QUANTIFICATION OF AMINO ACIDS IN BREATH

As stated above, the great sensitivity achieved by SESI-HRMS in the analysis of vapors, which can reach the low pptv range, allows the determination of not only volatile and semivolatile plasma components, but also low volatility compounds if present at sufficiently high concentrations. To check whether this is the case for PFAA, the expected breath concentrations were calculated from known plasma concentrations and tabulated Henry's constant values for aqueous solutions (**28**). Results (**Table 1**) suggest that some amino acids may be detectable in breath by SESI-HRMS because they

reach breath concentrations above the limit of detection of this technique. Based on this, breath SESI mass spectra were searched for amino acid signals (amino acids are expected to show up as protonated molecules, $[M+H^+]$), based on their accurate mass. Up to 8 amino acids, including those suggested by the calculations presented in **Table 1**, were clearly detected in breath of all individuals studied (**Fig. 2**). Their identification was not only supported by the mass accuracy of the instrument (below ±5 ppm), but also further strengthened by obtaining tandem MS spectra. In all cases, a loss of formic acid was found, typical of amino acids, which further confirms that the traces shown in **Fig. 2** correspond to the target compounds.

Table 1.

Collapse inline | View popup

Predicted concentrations of amino acids in breath.

Amino acid	Formula	$[M+H]^+$	Blood, μmol/L	K_H,[a] $mol \cdot L^{-1} \cdot atm^{-1}$	Breath, pptv
A	$C_3H_7NO_2$	90.0549	419	6.00E+07	6.98
L	$C_6H_{13}NO_2$	132.1019	160	2.00E+07	8.00
G	$C_2H_5NO_2$	76.0393	236	9.00E+07	2.62
R	$C_6H_{14}N_4O_2$	175.1189	89	1.00E+17	8.90E-10
S	$C_3H_7NO_3$	106.0499	114	4.00E+12	2.85E-05
Q	$C_5H_{10}N_2O_3$	147.0764	645	1.00E+13	6.45E-05
N	$C_4H_8N_2O_3$	133.0607	49	1.00E+13	4.90E-06

[a] See Sander (28).

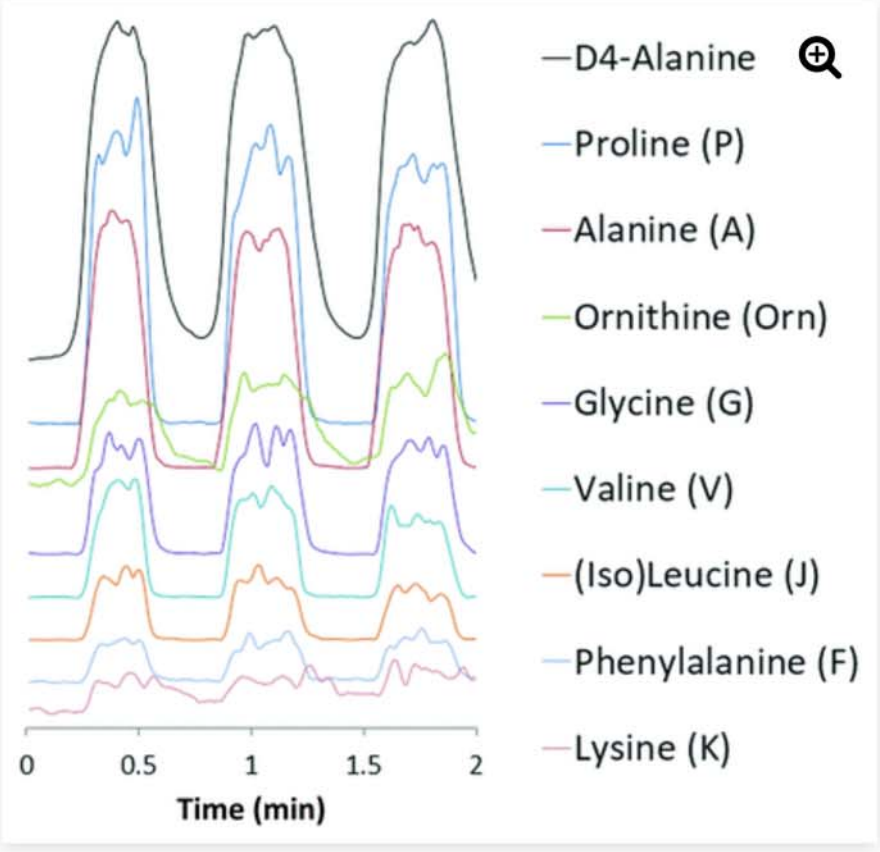

Fig. 2. **Download figure | Open in new tab | Download powerpoint**

SESI-HRMS traces for 8 amino acids in 3 consecutive exhalations.
Only the SESI-HRMS flow was recorded and the procedure was well tolerated by all participants. The trace for the internal standard (D4-A) has been scaled down.

Quantification of vapor concentration by SESI-HRMS is challenging, especially for low-volatility compounds, because of the difficulty of generating known vapor concentrations from chemical standards. Recently, Aernecke et al. have proposed a method (**35**) for quantifying this kind of compound based on the Clausius–Clapeyron equation. This method was applied here to quantify alanine in breath from 9 different participants (**Fig. 1**), which resulted in breath concentrations from 4 to 24 pptv (**Fig. 1**, last chart) confirming, in this way, our initial hypothesis, because breath concentration of alanine seems to be in line with predictions in **Table 1**. The limit of detection for this method, calculated as 3 times the signal-to-noise ratio, was found to be 0.6 pptv.

CORRELATION BETWEEN BREATH SIGNAL AND PFAA CONCENTRATION

It is known that venous blood concentration and breath concentration for a given metabolite are directly connected, which supports the use of breath analysis as a surrogate for plasma measurements (**36**, **37**). To check this correlation, Passing–Bablok regression curves were built for each amino acid using data from the training set (25 calibration points). These curves (see online Supplemental Table 2) confirmed a high correlation between breath signals and PFAA concentration (quantified by IE-HPLC) with Pearson's *r* and Spearman's ρ values above 0.85. Variability was checked for breath signals by repeated measurements and compared with IE-HPLC method (see online Supplemental Table 3). Values below 10% were found for all amino acids.

To validate these results, signals obtained from the validation set were converted to concentrations by means of Passing–Bablok calibration curves and compared to concentrations obtained by the gold standard IE-HPLC method. Results, as Bland–Altman plots, are shown in **Fig. 3**.

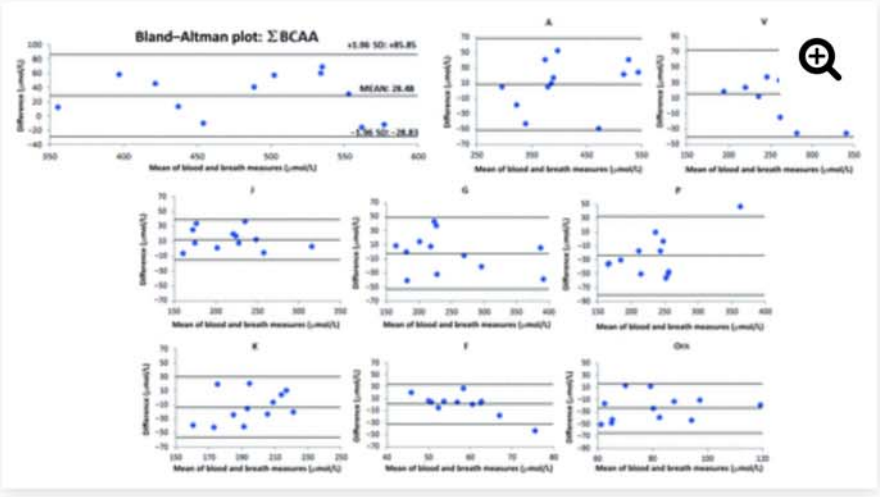

Fig. 3. **Download figure | Open in new tab | Download powerpoint**

Bland–Altman and correlation plots for breath and plasma concentrations of PFAAs.

It should be emphasized that the deviation between the 2 methods for the joint determination of V, L, and I (Σ BCAA, **Fig. 3**) was low (+5.9%) with an SE of ±12%. Regarding individual amino acids (**Fig. 3**), deviations were always below 10% with errors <25%, with the exception of F and Orn, that showed errors higher of 50%. This may be the result of their breath concentrations being too close to the limit of detection of the SESI-HRMS instrumentation and some concomitant biological factors that jeopardize the usefulness of the breath test for the quantification of these 2 amino acids. Generally, detected differences between the 2 measurement methods (IE-HPLC vs SESI-HRMS) were smaller than intraindividual differences for all amino acids (**29**).

LONGITUDINAL MEASUREMENTS OF AMINO ACIDS DURING 12 h

To show the capabilities of the method developed, longitudinal measurements of PFAA by SESI-HRMS breath analysis were run for 3 different participants during 12 h, excluding F and Orn. It is noted that measuring amino acids in exhaled breath is conveniently done and the noninvasiveness presents significant advantages over drawing blood for such time-dependent studies that require frequent sampling.

Measurements were started with participants in the fasting state and included breakfast and lunch. Results are shown in **Fig. 4**. Individual and grouped amino acids showed similar results, starting with high concentrations in fasting conditions that decreased after meals. Similar profiles were found by Fernstrom et al. (**38**) for medium protein diets by analyzing plasma samples.

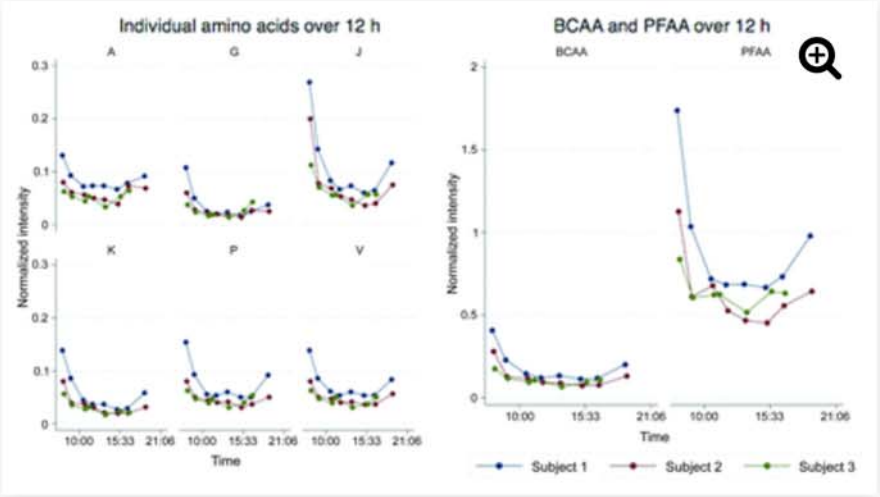

Fig. 4. **Download figure | Open in new tab | Download powerpoint**

Longitudinal amino acid profiles in 3 participants over 12 h.
Signals were normalized against D4-alanine.

Discussion

In this work, a novel method for quantifying amino acids in the human exhalome has been presented. This approach is distinct from traditional laboratory based blood plasma analytics in several important

aspects. In direct comparison with the current gold standard IE-HPLC, amino acid analysis in the exhalome via SESI-MS offers a noninvasive, less time-consuming, and less complex alternative. Furthermore, this technique theoretically has the potential to become a fully automated real-time process. Most importantly, traditional offline measurements of amino acids (i.e., IE-HPLC) suffer from preanalytic disruptions e.g., diverse supply chain management issues, storage/transport chain problems, and human factors influencing sample acquisition. Although this study was not designed to assess these issues, we suggest that exhalome analysis is characterized by much simpler (and more robust) preanalytics by allowing sample handling steps to be skipped as a result of its real-time computation of the results at comparable quality.

POTENTIAL APPLICATIONS OF SESI-MS

Generally, a method for amino acid analysis should have the following properties: (*a*) high sensitivity to identify amino acids present even at very low concentrations, (*b*) high specificity to enable distinction from interfering compounds, and (*c*) accuracy and high test–retest reliability to allow for longitudinal monitoring strategies for therapies. The current data of this proof-of-principle study suggest that SESI-MS is especially powerful in its accuracy and high test–retest reliability. It still has problems with amino acids with a low plasma concentration and/or a very low volatility (i.e., F and Orn) generating data with standard errors higher than 50% when compared to plasma analysis. Not a single participant with an IE-HPLC result in the reference interval showed an abnormal exhalome analysis result. Because this study was limited to healthy participants and did not include patients with known metabolic diseases, it is unknown whether the current technique is sufficiently accurate to diagnose patients with metabolic diseases, especially for diseases that result in abnormally low plasma concentrations of some amino acids. It should be noted, however, that most metabolic diseases are associated with abnormally increased rather than decreased concentrations of amino acids in blood, e.g., phenylketonuria and hyperphenylalaninemias (increased F); maple syrup urine disease (increased *a*lle); lactic acidosis, pyruvate metabolisms disorders and in mitochondriopathies (increased alanine); or propionic and methylmalonic acidemias (increased G). In these disorders, the corresponding amino acids can easily reach concentrations in the mmol/L range. Although SESI-MS has the potential to diagnose these diseases, the question remains of whether it will be sensitive enough to detect pathological levels of notoriously low abundance and/or less volatile amino acids like citrullin, *a*lle, argininosuccinate, or homocysteine, which are specific markers of some metabolic disorders.

We conclude that possible areas of clinical application of the current technique include (*a*) monitoring of disease activity and treatment/dietary compliance in patients; (*b*) large epidemiological studies investigating amino acid profiles (**3**); and (*c*) experimental studies for the identification of new pathways and biomarkers of disease. For example, based on this study, the monitoring of dietary compliance in patients suffering from phenylketonuria (a disease affecting 1 in 8000 individuals, who need regular monitoring of their blood F concentration) with SESI-MS seems like a promising and feasible clinical application.

PHYSIOLOGICAL CONSIDERATIONS

The described method also raises questions about the underlying physiological principles. It is unclear whether these results can be generalized to patients of different ages, patients with comorbidities, and children. Furthermore, breathing dynamics, temperature, and concentration gradient along the respiratory tree may influence the results the same way they do in traditional sample acquisition (temperature, blood stasis, venous/arterial gradient etc.). All these factors may contribute to an alteration of the final results. It is important to keep in mind that exhaled breath concentrations of substances only represent a proxy of the plasma concentration. Furthermore, there is evidence that the end-tidal breath concentration of substances and the underlying alveolar levels may be not identical, a factor which may have been revoked by data from our training set (**39**). In the light of these unknowns, our results were surprisingly accurate and allowed longitudinal measurements of high quality. Future studies are needed to confirm and expand the possible applications and performance characteristics of this method. For example, we measured only free amino acid patterns of blood plasma. These blood plasma patterns correlate strongly with those of muscles and erythrocytes, therefore new insights into human physiology with this technology could be hypothesized (**40**).

LIMITATIONS OF THE STUDY

This study comes with the limitations of a proof-of-principle study, namely, a small sample size, the lack of established performance characteristics for the novel technology (e.g., interday imprecision, trueness, specificity, interferences), and the fact that the remaining uncertainties between the 2 methods were not fully elucidated. Thus, further research is needed to address the remaining inaccuracies between the 2 methods with a focus on preanalytics for the IE-HPLC method and performance characteristics. Inconsistencies within the preanalytic part of the IE-HPLC method could partly explain small disagreements between the 2 methods. Amino acids are subject to degradation when they leave the body and to a certain extent even at freezing temperatures. For example, arginase released by the erythrocytes can convert plasma arginine into Orn, which would artificially increase the concentration of the latter amino acid. This is supported by the fact that Orn measurements by SESI-MS are biased negatively from the IE-HPLC measurements (−33%). Much more robust preanalytics for the SESI-MS measurements or the fact that real-time exhalome analysis skips downstream sample handling steps entirely supports our hypothesis that SESI-MS measurements reflect true amino acid concentrations well.

In conclusion, it has been shown for the first time that amino acids can be quantified in human breath in real time, and that their concentrations correlate with plasma concentrations. These results suggest that this diagnostic method has the potential to become an alternative to the traditional determination of amino acids in plasma. Although SESI-HRMS is still under development, it may establish itself as a useful and efficient broad-spectrum clinical method, which is capable of automation, unlimited iterations, and parallel measurement of other substances. The noninvasive approach combined with real-time analysis that skips traditional preanalytic obstacles (i.e., sample storage/treatment), suggest a broad area of clinical applications in the future.

Footnotes

- † Diego García-Gómez and Thomas Gaisl contributed equally to the work, and both should be considered as first authors.
- 6 Nonstandard abbreviations:

  PFAA,
  plasma free amino acids;

  BCAA,
  branched-chain amino acids;

  IE-HPLC,
  ion-exchange high-performance liquid chromatography;

  HRMS,
  high-resolution mass spectrometry;

  SESI,
  secondary electrospray ionization;

  pptv,
  parts-per-trillion by volume.

- **Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*
- **Authors' Disclosures or Potential Conflicts of Interest:** *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*
- **Employment or Leadership:** R. Zenobi, E.T.H. Zurich.
- **Consultant or Advisory Role:** None declared.
- **Stock Ownership:** None declared.
- **Honoraria:** None declared.
- **Research Funding:** Swiss National Science Foundation Grant.
- **Expert Testimony:** None declared.
- **Patents:** None declared.
- **Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, and final approval of manuscript.

Received for publication February 27, 2016.

Accepted for publication June 10, 2016.

References

1.Miyagi Y, Higashiyama M, Gochi A, Akaike M, Ishikawa T, Miura T, et al. Plasma free amino acid profiling of five types of cancer patients and its application for early detection. *PLoS One* 2011;**6**:e24143.

2.Shingyoji M, Iizasa T, Higashiyama M, Imamura F, Saruki N, Imaizumi A, et al. The significance and robustness of a plasma free amino acid (PFAA) profile-based multiplex function for detecting lung cancer. *BMC Cancer* 2013;**13**:77.

3.Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. *Nat Med* 2011;**17**:448–53.

4.Kume S, Araki S, Ono N, Shinhara A, Muramatsu T, Araki H, et al. Predictive properties of plasma amino acid profile for cardiovascular disease in patients with type 2 diabetes. *PLoS One* 2014;**9**:e101219.

5.Yamakado M, Nagao K, Imaizumi A, Tani M, Toda A, Tanaka T, et al. Plasma free amino acid profiles predict four-year risk of developing diabetes, metabolic syndrome, dyslipidemia, and hypertension in Japanese population. *Sci Rep* 2015;**5**:11918.

6.Kawai N, Mori M, Noguchi Y, Yamakado M, Tezuka T, Sakuma M, et al. Plasma free amino acid (PFAA) profile is a potential blood-based biomarker for Alzheimer's disease. *Alzheimers Dement* 2014;**10**:4–279.

7.Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat Rev Endocrinol* 2014;**10**:723–36.

8.McCormack SE, Shaham O, McCarthy MA, Deik AA, Wang TJ, Gerszten RE, et al. Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. *Pediatr Obes* 2013;**8**:52–61.

9.Newgard CB. Interplay between lipids and branched-chain amino acids in development of insulin resistance. *Cell Metab* 2012;**15**:606–14.

10.Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat Med* 2014;**20**:1193–8.

11.Martinez-Lozano Sinues P, Zenobi R, Kohler M. Analysis of the exhalome: a diagnostic tool of the future. *Chest* 2013;**144**:746–9.

12.Li X, Martinez-Lozano Sinues P, Dallmann R, Bregy L, Hollmen M, Proulx S, et al. Drug pharmacokinetics determined by real-time analysis of mouse breath. *Angew Chem Int Ed Engl* 2015;**54**:7815–8.

13.Martinez-Lozano P, Zingaro L, Finiguerra A, Cristoni S. Secondary electrospray ionization-mass spectrometry: breath study on a control group. *J Breath Res* 2011;**5**:016002.

14.Martinez-Lozano Sinues P, Tarokh L, Li X, Kohler M, Brown SA, Zenobi R, Dallmann R. Circadian variation of the human metabolome captured by real-time breath analysis. *PloS One* 2014;**9**:e114422.

15.Sinues PM-L, Kohler M, Zenobi R. Monitoring diurnal changes in exhaled human breath. *Anal Chem* 2013;**85**:369–73.

16.Garcia-Gomez D, Bregy L, Barrios-Collado C, Vidal-de-Miguel G, Zenobi R. Real-Time High-Resolution Tandem Mass Spectrometry Identifies Furan Derivatives in Exhaled Breath. *Anal Chem* 2015;**87**:6919–24.

17.Garcia-Gomez D, Bregy L, Nussbaumer-Ochsner Y, Gaisl T, Kohler M, Zenobi R. Detection and quantification of benzothiazoles in exhaled breath and exhaled breath condensate by real-time secondary electrospray ionization-high-resolution mass spectrometry and ultra-high performance liquid chromatography. *Environ Sci Technol* 2015;**49**:12519–24.

18.Martinez-Lozano Sinues P, Meier L, Berchtold C, Ivanov M, Sievi N, Camen G, et al. Breath analysis in real time by mass spectrometry in chronic obstructive pulmonary disease. *Respiration* 2014;**87**:301–10.

19.Schwarz EI, Martinez-Lozano Sinues P, Bregy L, Gaisl T, Garcia Gomez D, Gaugg MT, et al. Effects of CPAP therapy withdrawal on exhaled breath pattern in obstructive sleep apnoea. *Thorax* 2016;**71**:110–7.

20.Amann A, Costello Bde L, Miekisch W, Schubert J, Buszewski B, Pleil J, et al. The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva. *J Breath Res* 2014;**8**:034001.

21.Smith D, Spanel P, Herbig J, Beauchamp J. Mass spectrometry for real-time quantitative breath analysis. *J Breath Res* 2014;**8**:027101.

22.Jordan A, Haidacher S, Hanel G, Hartungen E, Mark L, Seehauser H, et al. A high resolution and high sensitivity proton-transfer-reaction time-of-flight mass spectrometer (PTR-TOF-MS). *Int J Mass Spectrom* 2009;**286**:122–8.

23.Huanwen Chen AW, Weihua Zhang, Renato Zenobi. Rapid in vivo fingerprinting of nonvolatile compounds in breath by extractive electrospray ionization quadrupole time-of-flight mass spectrometry. *Angew Chem Int Ed Engl* 2006;**119**:586–9.

24.Vidal-de-Miguel G, Macia M, Pinacho P, Blanco J. Low-sample flow secondary electrospray ionization: improving vapor ionization efficiency. *Anal Chem* 2012;**84**:8475–9.

25.Armstrong MD, Stave U. A study of plasma free amino acid levels. II. Normal values for children and adults. *Metabolism* 1973;**22**:561–9.

26.Mendez JA, Fernandez-Sanmamed AL, Gomez-Holgado MS, Fernandez-Rodriguez F. Age-related reference values for plasma amino acids in a Spanish population measured by gas chromatography-mass spectrometry. *J Pediatr Endocrinol Metab* 2013;**26**:333–41.

27.Qu J, Wang Y, Luo G, Wu Z, Yang C. Validated quantitation of underivatized amino acids in human blood samples by volatile ion-pair reversed-phase liquid chromatography coupled to isotope dilution tandem mass spectrometry. *Anal Chem* 2002;**74**:2034–40.

28.Sander R. Compilation of Henry's law constants (version 4.0) for water as solvent. *Atmos Chem Phys* 2015;**15**:4399–981.

29.Scriver CR, Beaudet AL, Sly WS, Valle DScriver CR. Amino acids. In: Scriver CR, Beaudet AL, Sly WS, Valle D editors. *The metabolomic and molecular bases of inherited disease*, 8th ed. New York: McGraw-Hill Medical; 2001. Part 8.

30.Sukul P, Trefz P, Schubert JK, Miekisch W. Immediate effects of breath holding maneuvers onto composition of exhaled breath. *J Breath Res* 2014;**8**:037102.

31.Thekedar B, Oeh U, Szymczak W, Hoeschen C, Paretzke HG. Influences of mixed expiratory sampling parameters on exhaled volatile organic compound concentrations. *J Breath Res* 2011;**5**:016001.

32.Wu C, Siems WF, Hill HH Jr.. Secondary electrospray ionization ion mobility spectrometry/mass spectrometry of illicit drugs. *Anal Chem* 2000;**72**:396–403.

33.Spackman DH, Stein WH, Moore S. Automatic recording apparatus for use in the chromatography of amino acids. *Anal Chem* 1958;**30**:1190–206.

34.Bablok W, Passing H, Bender R, Schneider B. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. *J Clin Chem Clin Biochem* 1988;**26**:783–90.

35.Aernecke MJ, Mendum T, Geurtsen G, Ostrinskaya A, Kunz RR. Vapor pressure of hexamethylene triperoxide diamine (HMTD) estimated using secondary electrospray ionization mass spectrometry. *J Phys Chem A* 2015;**119**:11514–22.

36.King J, Unterkofler K, Teschl G, Teschl S, Koc H, Hinterhuber H, Amann A. A mathematical method for breath gas analysis of volatile organic compounds with special emphasis on acetone. *J Math Biol* 2011;**63**:959–99.

37.O'Hara ME, Clutton-Brock TH, Green S, Mayhew CA. Endogenous volatile organic compounds in breath and blood of healthy volunteers: examining breath analysis as a surrogate for blood measurements. *J Breath Res* 2009;**3**:027005.

38.Fernstrom JD, Wurtman RJ, Hammarstrom-Wiklund B, Rand WM, Munro HN, Davidson CS. Diurnal variations in plasma concentrations of tryptophan, tryosine, and other neutral amino acids: effect of dietary protein intake. *Am J Clin Nutr* 1979;**32**:1912–22.

39.Amann A, Smith DKing J, Koc H, Unterkofler K, Teschl G, Teschl S, Mochalski P, et al. Physiological modeling for analysis of exhaled breath. In: Amann A, Smith D, editors. *Volatile biomarkers: non-invasive diagnosis in physiology and medicine*. Amsterdam: Elsevier B.V.; 2013. p 26–46.

40.Filho JC, Bergstrom J, Stehle P, Furst P. Simultaneous measurements of free amino acid patterns of plasma, muscle and erythrocytes in healthy human subjects. *Clin Nutr* 1997;**16**:299–305.

Options

Home
About
Articles
Info for
Resources
Abstracts
Submit
Contact

Other Publications

The Journal of Applied Laboratory Medicine